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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The overall aim of this research is to use a recombinant multiply deleted genomic herpes simplex virus type-1 (HSV-1) based gene transfer vector to test the hypothesis that gene transfer of the glial derived neurotrophic factor (GDNF) or bcl-2 can slow or prevent the apoptotic death of nigral neurons in animal models of PD, and that expression of tyrosine hydroxylase (TH) can improve symptoms.

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4. Introduction.

The overall aim of these studies is to test herpes simplex virus (HSV)-based gene transfer vectors in rodent models of Parkinson's disease (PD) in order to develop novel therapeutic agents that might be used to slow the progression or reverse the course of idiopathic PD. The Specific Aims in the initial proposal were to create genomic HSV-based vectors to express the anti-apoptotic peptide Bcl-2 and the glial cell derived neurotrophic factor (GDNF), to examine the effects of these vectors in preventing characteristic neurodegeneration in the substantia nigra (SN) in the 6 hydroxydopamine (6-OHDA) and MPTP models of PD in rodents, and to compare the time course of the protective effect produced by vectors in which transgene expression was driven by the transiently active human cytomegalovirus immediate early promoter (HCMV IEp) or HSV-based latency active promoter (LAP2) element.

5. Body.

In the first two years of these funded studies we have found that a genomic HSV vector expressing the anti-apoptotic peptide Bcl-2 can protect neurons of the SN from 6-OHDA toxicity [1], that an HSV-vector expressing GDNF also has a protective effect [2], and that the Bcl-2-expressing and GDNF-expressing vectors delivered together provide an additive effect [2] in protecting SN neurons from cell death induced by 6-OHDA, in preserving expression of the neurotransmitter-synthesizing enzyme tyrosine hydroxylase (TH) and in the dopaminergic neurons of the SN. This additive effect is different from the response we observed in a different model of neuronal injury (proximal spinal root avulsion) where we found that the Bcl-2 expressing vector protected lesioned motor neurons from degeneration [3] but does not preserve the neurotransmitter phenotype (choline acetyltransferase (ChAT) expression) of the lesioned cells [3]. In that model the GDNF-expressing vector has a similar effect on cell survival and does not preserve ChAT expression [4]. However, the two vectors delivered together acted synergistically to preserve ChAT expression in lesioned cells [4]. These studies provided proof-of-principle evidence that HSV-mediated gene transfer may be used to protect neurons of the SN from 6-OHDA induced neurotoxicity, and have been published. Each of thes studies employed an experimental paradigm in which the vectors were injected into the striatum 1 week prior to 6-OHDA lesioning, and cell survival, neurotransmitter expression and behavioral effects determined 2 weeks after lesioning.

In order to determine whether vector mediated gene transfer might provide a more prolonged cell protection, in the pas year we carried out experiments to examined whether HSV-mediated gene transfer of GDNF 30 days prior to lesioning might provide a protective effect. For these studies we used the genomic HSV vector (DHGD) that expresses GDNF under the control of the human cytomegalovirus immediate early promoter (HCMV IEp) and which was employed in our published studies described above, and constructed two new GDNF-expressing vectors; the first (QL2GD) with GDNF coding sequence placed under the control of the HSV latency promoter (LAP2), and the second (QL2HGD) with the GDNF coding sequence placed under the control of a promoter containing both LAP2 and HCMV elements.

Construction of vectors QL2GD and QL2HGD. QL2GD and QL2HGD were constructed in the same genetic backbone vector (QOZHG) used for creation of QHGD. For QL2GD a LAP2 driven expression construct targeting the Ul41 locus was created by inserting a BglII to BamHI fragment containing LAP2. The base LAT PK plasmid contains the Pst to KpnI fragment of the LAP region in pUC19. First, the PpuMI site in plasmid LAT PK was converted into a BamHI site by digesting with PpuMI, treating with Klenow polymerase, and ligating with a BamHI linker thus creating plasmid LAT PKB. The HinDIII site in plasmid LAT PKB was similarly converted into a BglII site creating plasmid LAP PKBHB. The BglII to BamHI LAP2 fragment from plasmid LAT PKBHB (640bp) was inserted into the unique BamHI site of plasmid pUL41 (HinDIII to NotI of the Ul41 region in pBSSK+) containing the BGH polyadenalyation element thus creating plasmid p41L2. To create the Ul41 targeting plasmid containing a chimeric LAP2/HCMV promoter, the BglII to BamHI fragment containing the HCMV promoter from plasmid pRC2 (Invitrogen) was then cloned into the unique BamHI site of plasmid p41L2 creating plasmid p41L2H. Thus both Ul41 targeting constructs contain Ul41 sequences flanking the promoter (LAP2 or LAP2/HCMV) followed by a unique BamHI site preceding a BGH polyA element. The BamHI fragment containing the GDNF coding sequence was inserted into the BamHI site of the appropriate targeting plasmid creating plasmids p41L2GD and p41L2HGD. These plasmids were contransfected with QOZHG viral DNA and plaques that did not sating blue (clear) upon X-Gal staining were identified and isolated by three rounds of single plaque purification. The genomic structure of QL2GD and QL2HGD were confirmed by Southern blotting of digested viral DNA (HinDIII and EcoRV) probed with GDNF or Ul41 sequences. Expression of GDNF from the purified vectors was confirmed by ELISA (Promega) on supernatant two days after infection of Vero or differentiated PC12 cells at an MOI of 3. Vector stocks were prepared by our standard protocol and purified on a continuous 30% Nycodenz (Amersham) gradient. Isolated vector bands were further concentrated by diluting with PBS, pelleted by centrifugation, and resuspended with 5% Nycodenz.. Vector stocks were aliquoted, stored at –80C and titered on complementing 7b cells.

Transgene expression in vivo. We examined the expression of GDNF in substantia at 12 days and 30 days following vector inoculation. 2 μl containing 3 x 10^6 pfu of DHGD, QL2GD or QL2HGD was injected stereotactically into the SN following our previously published protocol [1], and the amount of GDNF expressed determined by Western blot of protein isolated from freshly dissected SN. In agreement with the results we have previously reported following transduction of sensory ganglia with a nerve growth factor-expressing HSV vector [5] DHGD produced high levels of expression at 12 days post inoculation (PI), but by 30 days expression had fallen to barely detectable levels (Figure 1, HCMV). QL2DG transduction of SN produced a low level of expression 12 days PI, but by 30 days expression had increased substantially (Figure 1, LAP2). The LAP2/HCMV hybrid promoter element produced an intermediate level of expression at both 12 days and 30 days PI, less than HCMV but greater than LAP2 at 12 days, and less than LAP2 but greater than HCMV at 30 days (Figure 1, LAP2/HCMV).

QL2GD but not DHGD or QL2HGD protect DA neurons from 6-OHDA toxicity. The protection of the nigral DA neurons was evaluated by counting the numbers of FG-labeled neurons in the SN bilaterally. In control animals (PBS- and DHZ-injected), intrastriatal injection of 6-OHDA (5 μl) resulted in the loss of more than 80% of FG-labeled neurons compared to the contralateral unlesioned side. Injection of QL2GD 30days prior to lesioning resulted in a substantial increase in the number of surviving FG-labeled cells in lesioned striatum (58% of contralateral side, $P < .01$ compared to DHZ.4, DHGD, or QL2HGD, Figures 2 and 3). Neither DHGD nor QL2HGD had any effect on cell survival in SN (Figures 2 and 3).

QL2GD but not DHGD or QL2HGD preserves TH expression in 6-OHDA lesioned neurons. Preservation of neurotransmitter phenotype was determined by examining the survival of TH-immunoreactive (TH-IR) cells in the lesioned compared to intact SN in control and vector-treated animals. The loss of TH-IR cell bodies (approximately 60%) compared to the uninjected contralateral side was not as great as the loss of FG-labeled neurons (approximately 80%). This reflects the fact that while all the terminals of FG-labeled cells were exposed to 6-OHDA which was injected at the same coordinates a week after the FG, TH-IR cells include a population that project to uninjected (i.e. unlesioned) regions of striatum, and were therefore not affected by 6-OHDA. Injection of QL2GD 30days prior to lesioning increased the number of TH-IR cells surviving in lesioned striatum (75% of contralateral side, $P < .01$ compared to DHZ.4, DHGD, or QL2HGD, Figures 4 and 5). Neither DHGD nor QL2HGD injected 30 days prior to lesioning had any effect on TH-IR in the SN (Figures 4 and 5).

QL2GD did not reduce amphetamine-induced ipsilateral rotational behavior. Rats were injected with D-amphetamine (5 mg/kg body weight i.p.) 14 days after 6-OHDA lesioning (44 days after vector inoculation) and their behavior recorded for 90 min. Amphetamine-induced release of DA causes animals with a unilateral lesion of nigrostriatal DA system to turn toward the lesioned striatum. In control lesioned rats injected with the lacZ-expressing vector DHZ.4, ispilateral rotational behavior (approximately 5 turns/min) towards the lesioned hemisphere was observed. None of the GDNF expressing vectors injected 30 days prior to 6-OHDA had any impact on this behavioral measure (Figure 6).

Survival of TH-immunoreactive terminals in the striatum is not improved by vectors injected 30 days prior to lesioning. In order to further examine the pathophysiology underlying the failure to correct rotational behavior in the face of increased cell survival and preserved TH-IR in the SN, we examined the lesioned striatum. Animals injected with QL2GD showed a pattern of loss of striatal TH-IR (Figure 7) that was similar to the control (DHZ.4) and other GDNF-expressing vectors (DHGD and QL2HGD).

Discussion. We identified two important differences between the experiments described above and our previously reported work using the same vectors. The difference in time of vector inoculation (30 days prior to 6-OHDA lesioning in this study, 7 days prior to 6-OHDA lesioning in the previous studies) and promoter constructs employed (LAP2 and the LAP2-HCMV IEp fusion construct in addition to the HCMV IEp used in our previous work) was the focus of these studies. An unanticipated difference was found in the severity of the initial lesion in all of the

groups tested in this study. In prior experiments, vector control-injected lesioned animals suffered a 70% loss of cells in the SN measured by FG labeling (compared to contralateral) and a 40% loss measured by TH-IR. Although the 6-OHDA lesioning protocol used in the continuation studies was identical, cell death in the untreated animals in these experiments was greater (80% by FG, 65% by TH-IR). It seems not unlikely that the protection against neurodegeneration produced by the QL2GD which was readily detectable by FG labeling and TH-IR failed to correct the behavioral manifestation because the initial lesion was more severe, so that lesioned-treated animals had insufficient surviving TH-positive terminals to correct the behavioral abnormality. A repeat of these experiments is underway, as are studies in MPTP intoxicated mice in which the vector has been injected after the onset of MPTP intoxication.

6. Key Research Accomplishments

a. Expression of Bcl-2 and GDNF by HSV mediated gene transfer provides an additive effect in the PD model, and a synergistic effect in protecting other neurons form the effects of trauma.

b. The LAP2 promoter element if effective in driving prolonged expression of GDNF from an HSV gene transfer vector, in order to protect SN neurons from neurodegeneration, measured by survival of FG-labeled neurons and TH-IR cells in the SN.

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Presentations:

New Horizons IV Discoveries in Sight Conference, "Gene Transfer Using Herpes Simplex" Portland, OR, August, 2000

Legacy Health System, Chief of Research Lecture Series, "Gene Transfer to the Nervous System" Portland, OR, October, 2000

University of Pittsburgh, School of Medicine, Department of Endocrinology Research Conference, "Herpesvirus Vectors and Applications To Gene Therapy" Pittsburgh, PA, October, 2000

Mayo Clinic Guggenheim Molecular Medicine Program Seminar Series, "Application of Herpes Gene Vectors to the Treatment of Human Disease", Rochester, MN, October 2000

McGill University, Department of Human Genetics, "Design and Application of HSV Vectors to Nervous System Disease", Montreal, Canada, December 2000

Keystone Symposium—Gene Therapy 2001: A Gene Odyssey, "Applications of HSV Vectors to Nervous System Disease", Snowbird, UT, January 2001

University of Texas Health Science Center at San Antonio, "Design of HSV Gene Vectors for Treatment of Nervous System Disease", San Antonio, TX, January 2001

University of California San Francisco, Department of Neurology, "Modifying the structure and function of the nervous system by HSV-mediated gene transfer" San Francisco, CA, January, 2001

University of Massachusetts, Worcester, Department of Neurology, "Modifying the structure and function of the nervous system by HSV-mediated gene transfer" Worcester, MA, February, 2001

American Academy of Orthopaedic Surgeons 68th Annual Meeting, "Application of HSV Vectors to the Treatment of Pain", San Francisco, CA, March 2001

Cold Spring Harbor Laboratory Winter Biotechnology Conference, "Application of HSV Vectors to the Treatment of Pain", Cold Spring Harbor, NY, March 2001

Neurotoxin Exposure Treatment Research Program on Parkinson's Research, "Gene Transfer Studies of the Pathogenesis and Treatment of Parkinson's Disease", Potomac, MD, March 2001

Institute for Human Gene Therapy Research Seminar Series, "Design and Application of Herpesvirus Gene Vectors to the Treatment of Nervous System Diseases", Philadelphia, PA, April 2001

American Society for Microbiology, ASM Conference on Viral Gene Vectors: Molecular Biology, Design & Application to Gene Therapy, "Gene Therapy of Neurologic Disease with HSV Vectors" Banff, Alberta, Canada, April, 2001

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Glaucoma Foundation Eighth International Think Tank, "Preventing Cell Death with HSV-based vectors" New York, NY, July, 2001

Gene Therapy and Molecular Biology International Conference, Plenary Talk, "Modifying Neural Structure and Function with HSV-based vectors" Corfu, Greece, August, 2001

8. Conclusions

The experiments performed to date demonstrate that HSV-mediated gene transfer and expression of Bcl-2 and GDNF act additively to prevent degeneration of TH-IR neurons of the SN in the 6-OHDA model in rats. The effect can be prolonged by using the HSV LAP2 promoter in place of the HCMV promoter to drive transgene expression, but while inoculation of that vector (LAP2-GDNF) 30 days prior to 6-OHDA lesioning improves survival of the SN neuronal somata and TH-IR in those cells, it fails to improve survival of the axonal processes or have the appropriate behavioral effect. We have begun our studies of the MPTP model, in which the vector is injected during the course of neurodegeneration, and in the final year of this project will complete those studies, as well as examine the effect of co-administration of the HCMV-Bcl2 vector with the LAP2-GDNF vector in the 6-OHDA model.

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4. Natsume, A., et al., Bcl-2 and GDNF delivered by HSV-mediated gene transfer after spinal root avulsion provide a synergistic effect. J. Neurotrauma, in press.

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Figure Legends.

FIGURE 1. Expression of GDNF in SN 12 days and 30 days PI. Rats were injected with QL2GD (LAP2), DHGD (HCMV), or QL2HGD (LAP2/HCMV) into SN and the amount of GDNF in SN determined by Western blot of protein from homogenate of freshly dissected ipsilateral SN .

FIGURE 2. Survival of FG labeled neurons in SN. Animals were injected with the vector 30 days prior to 6-OHDA injection into striatum and sacrificed 2 wks after lesioning. The survival of neurons in SN was assessed by counts of the number of neurons retrogradely labeled by FG injected into striatum prior to lesioning. DHZ.4 – lacZ; DHGD – HCMV:GDNF; QL2GD - LAP2:GDNF; QL2HGD – LAP2-HCMV:GDNF.

FIGURE 3. Survival of FG labeled neurons in SN. The number of FG-labeled cells was counted in every fourth section. Only QL2GD protected SN neurons from degeneration ($P < .01$ by ANOVA compared to each of the other groups).

FIGURE 4. Survival of TH-IR neurons in SN. Animals were injected with the vector 30 days prior to 6-OHDA injection into striatum and sacrificed 2 wks after lesioning. DHZ.4 – lacZ; DHGD – HCMV:GDNF; QL2GD - LAP2:GDNF; QL2HGD – LAP2-HCMV:GDNF.

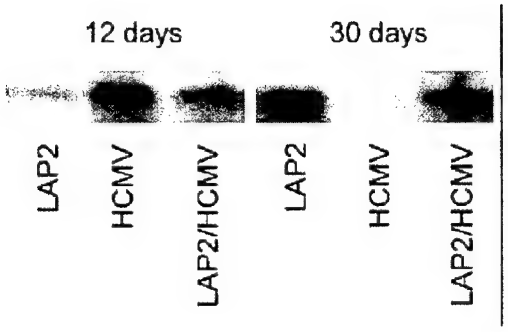
FIGURE 5. Survival of TH-IR neurons in SN. The number of TH-IR neurons surviving in SN was counted in every fourth section. Only QL2GD protected SN neurons ($P < .01$ by ANOVA compared to each of the other groups).

FIGURE 6. Ipsilateral rotation following injection of d-amphetamine was not prevented by any of the vectors.

FIGURE 7. TH IR in lesioned striatum was not significantly restored by QL2GD. TH-IR 14 days after 6-OHDA shows significant loss in the lesioned striatum from this animal injected with QL2GD. Similar results were found in animals injected with DHZ, DHGD and QL2HGD vectors.

FIGURES

FIGURE 1



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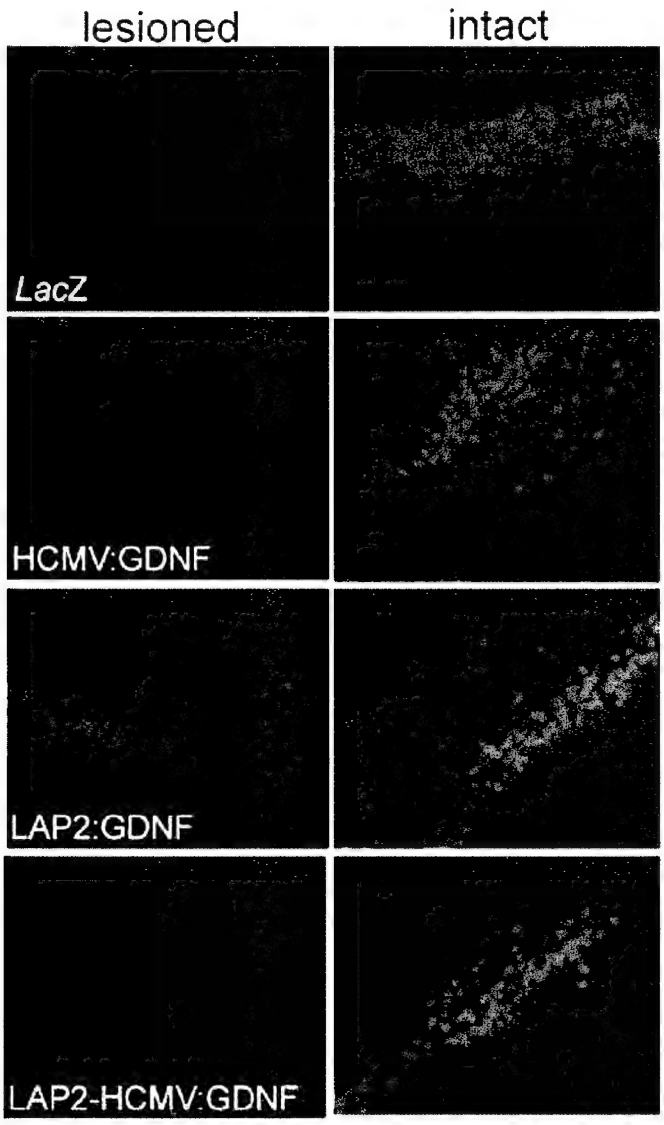

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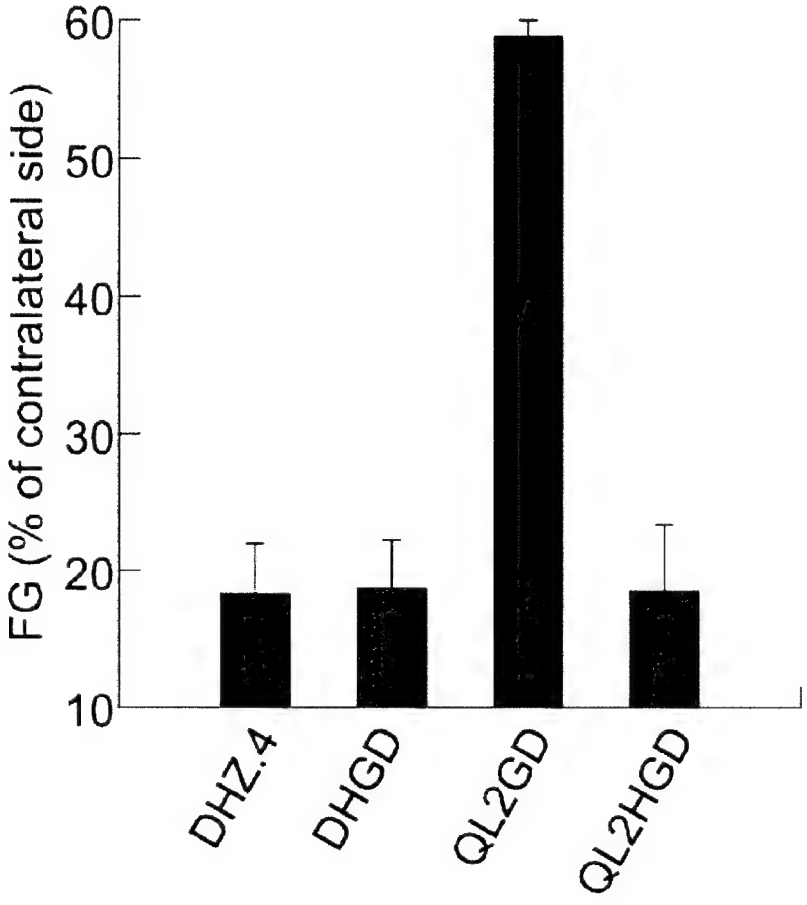
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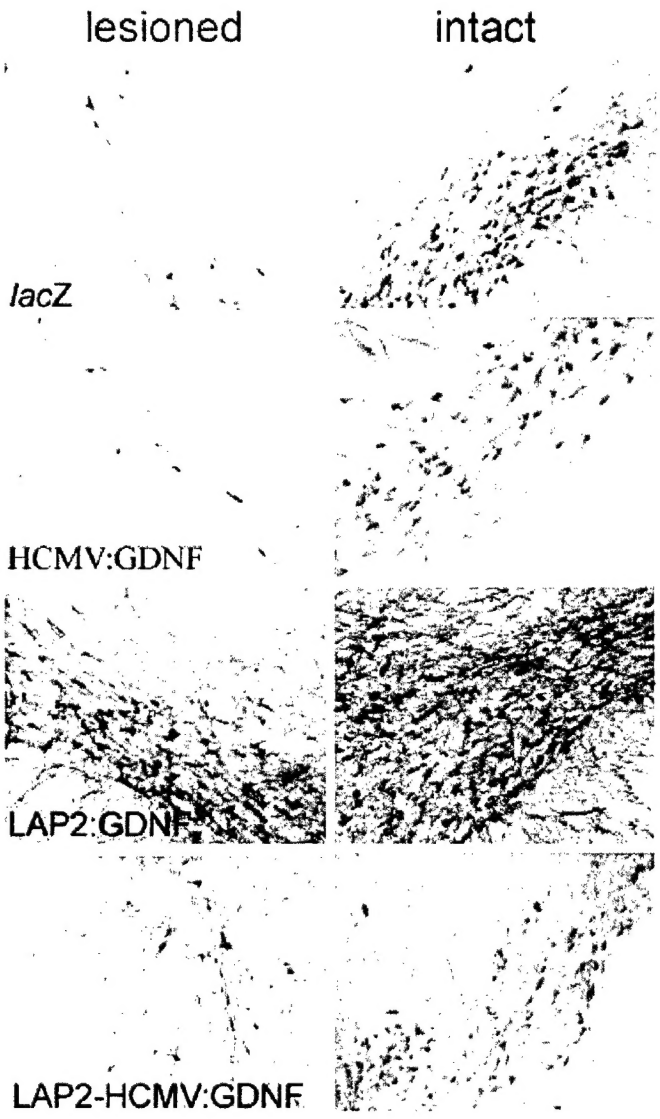
50
40
30
20
10
DHZ.4
DHGD
QL2GD
QL2HGD

FIGURE 4



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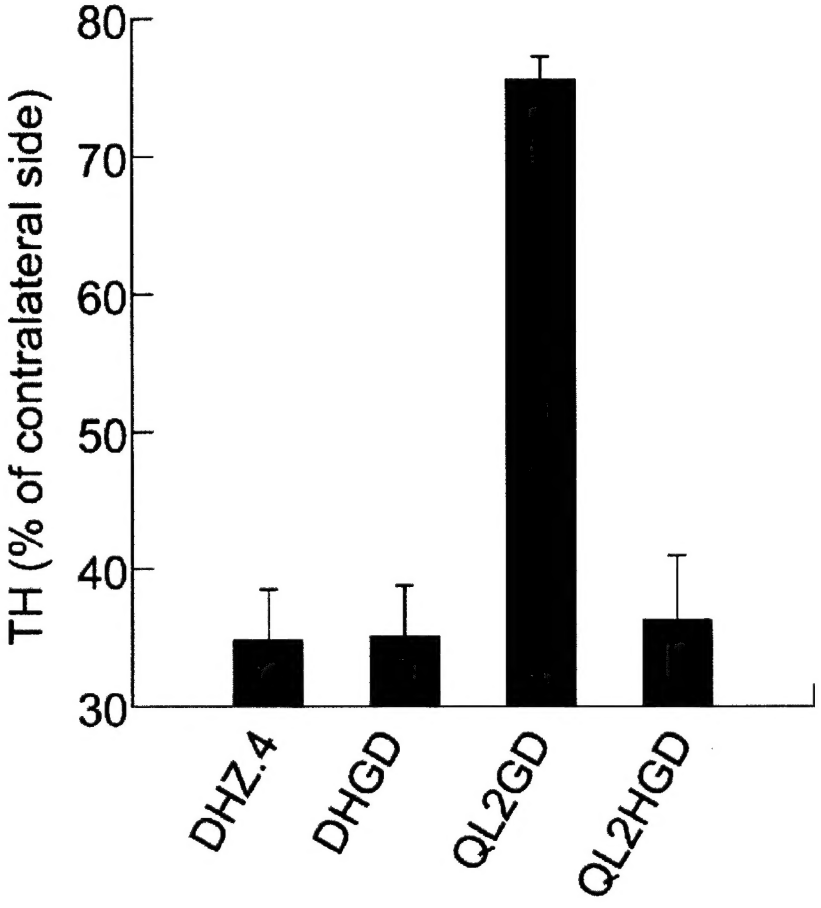
TH (% of contralateral side)
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70
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50
40
30
DHZ.4
DHGD
QL2GD
QL2HGD

FIGURE 6

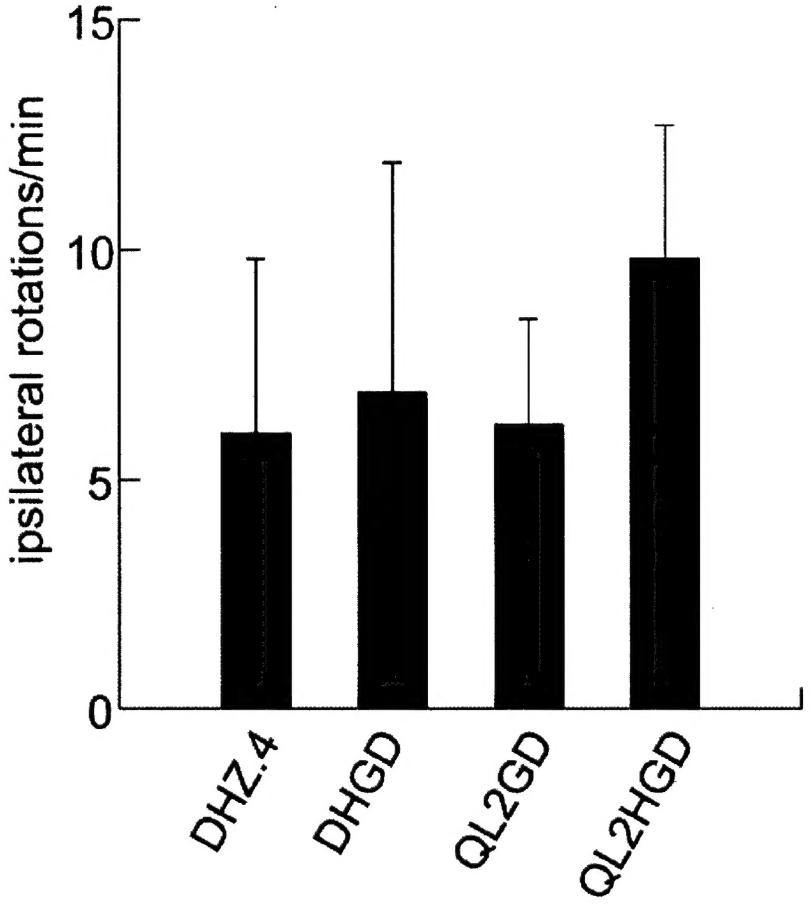

FIGURE 7